



Pulmonary, Gastrointestinal and Urogenital Pharmacology

M₃ muscarinic receptor antagonist bencycloquidinium bromide attenuates allergic airway inflammation, hyperresponsiveness and remodeling in miceRui Cao^{a,1}, Xin-Wei Dong^{a,1}, Jun-Xia Jiang^a, Xiao-Feng Yan^a, Jun-Shan He^a, Yang-Mei Deng^a, Fen-Fen Li^a, Meng-Jing Bao^a, Yi-Cheng Xie^a, Xiao-Ping Chen^b, Qiang-Min Xie^{a,*}^a Zhejiang Respiratory Drugs Research Laboratory of State Food and Drug Administration of China, Medical College of Zhejiang University, Hangzhou, China^b Jiashilianbo Medicine Science & Technique Co., Beijing 100080, China

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ABSTRACT

M₃ muscarinic receptors are localized on inflammatory cells, airway smooth muscle, and submucosal glands, known to mediate bronchoconstriction, mucus secretion, and airway remodeling. It is hypothesized bencycloquidinium bromide (BCQB), a novel M₃ receptor antagonist, might have potential effects on airway hyperresponsiveness, inflammation and airway remodeling in a murine model of asthma. Mice sensitized and challenged with ovalbumin developed airway inflammation. Bronchoalveolar lavage fluid was examined to determine the total and differential cell counts, and cytokine levels. Lung tissues were evaluated for cell infiltration, mucus hypersecretion, airway remodeling, and the expression of inflammatory biomarkers. Airway hyperresponsiveness was monitored by direct airway resistance analysis. Inhalation administration of BCQB significantly not only reduced ovalbumin-induced airway hyperresponsiveness comparing to methacholine, and prevented the ovalbumin-induced increase in total cell counts and eosinophil counts. Reverse transcriptase polymerase chain reaction analysis of whole lung lysates revealed that BCQB markedly suppressed ovalbumin-induced mRNA expression of eotaxin, IL-5, IL-4 and MMP-9, and increased mRNA expression of IFN- γ and TIMP-1 in a dose-dependent manner. Substantial IFN- γ /IL-4 (Th1/Th2) levels were recovered in bronchoalveolar lavage fluid after BCQB treatment. In addition, histological studies showed that BCQB dramatically inhibited ovalbumin-induced lung tissue eosinophil infiltration, airway mucus production and collagen deposition in lung tissues. Results reported in current paper suggest that M₃ receptors antagonist may provide a novel therapeutic approach to treat airway inflammation, hyperresponsiveness and remodeling.

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1. Introduction

Asthma is characterized by inflammation, airway remodeling and hyperresponsiveness (Global Initiative for Asthma (GINA), 2009). Upon simultaneous exposure to both allergic inflammation and viral infections, individuals who develop asthma with chronic obstructive pulmonary disease (COPD)-like inflammation, appear to be poorly responsive to β_2 -agonists (Kanazawa, 2006). Exposure to tobacco smoke has several effects in patients at risk for asthma. It increases the risk of allergic sensitization, which is associated with accelerated decline of lung function in people with asthma. In addition, it increases the severity of asthma, and render patients less responsive to treatment with inhaled (Chalmers et al., 2002; Lazarus et al., 2007) and systemic glucocorticosteroids (Chaudhuri et al., 2003), and

reduces the likelihood of asthma being controlled (Bateman et al., 2004). Patients who experience the above mentioned problems may respond better to anticholinergic agents.

Parasympathetic nerves provide the dominant autonomic innervation of airways. Muscarinic (M)₁, M₂, and M₃ receptor subtypes are expressed in airways and have relevant physiological roles. M₁ receptor subtypes are found in parasympathetic ganglia where they facilitate neurotransmission. M₂ receptors are localized prejunctionally on the postganglionic cholinergic nerve terminals and provide a functional negative feedback on acetylcholine release. M₃ receptors are localized on airway smooth muscle, submucosal glands, and blood vessels and mediate bronchoconstriction, mucus secretion, and vasodilatation (Minette and Barnes, 1990). In asthma and COPD, bronchoconstriction and mucus secretion is increased and the airways are hyperresponsive to contractile agents. These changes are due to increased parasympathetic nerve activity (Gross et al., 1989). The number and function of post-junctional M₃ muscarinic receptors in the airways are unchanged in asthma and COPD (Chiba and Misawa, 1995; Fryer and Wills-Karp, 1991; Schultheis et al., 1994; Fisher et al., 2004). Rather, it is the supply of acetylcholine to the postjunctional

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effector cells that is increased. The increase of acetylcholine release occurs because pre-junctional inhibitory M₂ muscarinic receptors on the parasympathetic nerves are dysfunctional (Coulson and Fryer, 2003).

Bencycloquidinium bromide (BCQB), chemically known as 3-[(2-cyclopentyl-2-hydroxy-2-phenyl) ethoxy]-1-methyl-1-azabicyclo [2,2,2] octane bromide, is a quaternary ammonium salt with anticholinergic properties (Zhou et al., 2009; Jiang et al., 2010). A previous pharmacokinetic study of this compound in rats, after intranasal administration of 3.0 mg/kg BCQB, demonstrated a long elimination half-life (11.7 ± 7.1 h) (Xu et al., 2007). However, a pharmacokinetic study in humans using nasal spray of BCQB 45 µg (Zhou et al., 2009) and in dogs via intratracheal injection of BCQB at the dose of 100, 200 and 400 µg (Jiang et al., 2010), did appear to have a shorter elimination half-life compared to rats. Results from radioligand receptor binding assays using membranes of Chinese hamster ovary (CHO) cells expressing human muscarinic receptors found that BCQB has high affinity and potency [(M₃>M₁>M₂) (see supplementary data)] on human M₃ receptor. This result indicates that BCQB is a selective M₃ receptor antagonist. However, the potential anti-inflammatory, anti-hyperreactive and anti-remodeling effects of selective M₃ receptor antagonists on allergen-induced effects in a mouse asthmatic model have not been described to date. Therefore, the aim of the present study is to investigate the effects of BCQB, on airway inflammation and compared effects to tiotropium bromide, a non-selective muscarinic receptor antagonist.

2. Materials and methods

2.1. Animals

Outbred female pathogen-free ICR mice (Laboratory Animal Center of Zhejiang University, China; Certificate No. SCXK 2007-0029) weighing 20 ± 2 g were studied in all experiments. All animals were housed in Plexiglas cages and kept on a 12/12 h light–dark cycle in temperature and humidity controlled rooms. Food was withheld 8 h before the experiments, with free access to water. Unless otherwise indicated in the text, standard laboratory food and water were provided *ad libitum*.

To sensitize the mice, 100 µg ovalbumin (Grade V, Sigma Chemical Co., St. Louis, MO) adsorbed on 2 mg alum aluminum hydroxide adjuvant was subcutaneously injected into footpad, neck, back, and groin on days 0 and 14. Control mice were injected with saline following the same protocol. All protocols described in this study were approved by the University of Zhejiang Committee for Animal Experimentation.

2.2. Study design

Mice were divided into six groups. To investigate the treatment effects of BCQB (Jiashilianbo Medicine Science & Technique Co., Beijing, China) on airway hyperresponsiveness, inflammation and remodeling, treatment groups were administered inhaled BCQB at 60, 120 and 240 µg/mL by a jet nebulizer (BARI Co. Ltd, Germany). Treatment was repeated daily from day 22 to day 28. An equal volume of saline inhalation was substituted for the BCQB in the model group and control group, respectively. Fifteen minutes after the treatment, mice were placed in a plastic box and challenged with aerosolized ovalbumin (10 mg/ml in saline) in the jet nebulizer for 30 min, which was also repeated daily from day 22 to day 28. The control group was treated with an equal volume of saline as previously described (Deng et al., 2006).

2.3. Measurement of airway hyperresponsiveness

The changes in airway resistance (R_L) and lung dynamic compliance (C_{dyn}) were used to evaluate the changes in airway function after intravenous methacholine (Sigma, St. Louis, USA). The

anesthetized and tracheostomized mice was assessed as previously described (Deng et al., 2006). Cumulative dose–effect curves were established and the provocation dose (PD) of methacholine which produced a 100% increase in lung resistance (PD₁₀₀) or a 25% fall in lung dynamic compliance (PD₂₅) was determined for each animal. These values were used as indices of airway responsiveness. Cumulative doses of 0.0625–0.5 mg/kg methacholine (i.v. via a jugular vein cannula) were administered for a period of 5 min per dose with lung function measurements taken between each dose.

2.4. Reverse transcriptase polymerase chain reaction

Total RNA was extracted from lung homogenates in 1 ml TRIzol (Takara Bio, Dalian, China) per the manufacturer's instructions. 1 µg aliquots of the total RNA was reverse transcribed to cDNA using reverse transcriptase moloney murine leukemia virus (RTase M-MLV, Takara Bio, Dalian, China) according to the manufacturer's instructions. The PCR amplification was performed in a total volume of 25 µl (1.25 U of LA Taq DNA polymerase; Takara, JP), 0.2 µM sense and antisense primers, and 400 µM dATP, dTTP, dGTP and dCTP. All PCR reactions were performed in a gradient thermal cycler PCR machine (Eppendorf, Germany). For PCR amplification, the following mouse-specific sense and antisense primers were used in Table 1. PCR samples and markers were separated in 1.5% agarose gels containing 0.5 µg/ml ethidium bromide. Photographs of UV-transilluminated ethidium bromide-stained agarose gels were visualized with an imaging system (UVP company limited, England). Results were expressed as a ratio of OD signal for eotaxin and cytokines to that for β-actin. All primers were checked against GenBank for selectivity.

2.5. Preparation of bronchoalveolar lavage fluids

Seven days after the ovalbumin challenge, mice were anaesthetized with urethane (2 g/kg, i.p.). Bronchoalveolar lavage fluids were obtained by cannulating the trachea and lavaging with phosphate buffer solution (PBS) containing 1% bovine serum albumin and 5000 IU/L heparin. Bronchoalveolar lavage fluids were centrifuged at $500 \times g$ for 10 min at 4 °C. The supernatant was stored at –80 °C. The pelleted bronchoalveolar lavage fluid cells were resuspended in PBS and the total number of leucocytes was counted in a Neubauer chamber. Two hundred cells from bronchoalveolar lavage fluids stained with Wright-Giemsa were differentiated under a light microscope. The total number of each cell type was determined by multiplying the percentage of each cell type by the total number of cells. The results were expressed as the number of each cell type in 1 ml of bronchoalveolar lavage fluids.

Table 1
The gene primers (5'–3'), annealing temperature and cycle.

Subjects	Gene primers	Annealing temperature (°C)	Cycle
Eotaxin	forward-AGAGGCTGAGATCCAAGCAG reverse-ACAGATCTCTTTGCCCAACCT	61	32
IL-4	forward-ACGGCAGAGAGCTAATTGATG reverse-ATGGTGGCTCAGTACTACGA	55	35
IL-5	forward-ATGACTGTGCTCTGTGCTGGAGC reverse-CTGTTTTCTGGAGTAACTGGGG	61	35
IFN-γ	forward-CCTCAGACTCTTTGAAGTCT reverse-CAGCGACTCTTTTCTCTT	58	35
MMP-9	forward-GTATGGTCGTGGCTCTAAGC reverse-AAAACCTCTTGGTCTGCGG	60	35
TIMP-1	forward-GACCACCTTATACCAAGCGTT reverse-GTCACTCTCCAGTTTGAAG	55.5	32
β-actin	forward-CTGTCCCTGTATGCTCTG reverse-ATGTACGACGCA TTTC	55	32

2.6. Enzyme linked immunosorbent assay

The frozen bronchoalveolar lavage fluids were thawed on ice and centrifuged at $2000 \times g$ for 60 min at 4°C . The concentrations of IL-4 and IFN- γ in bronchoalveolar lavage fluid supernatants were measured by sandwich ELISA using pair matched antibodies according to the manufacturer's instructions. Both ELISA kits were obtained from eBioscience. The limits of detection were as follows: IL-4, 4 pg/ml and IFN- γ , 15 pg/ml.

2.7. Tissue processing and histological analysis

Right lungs were taken from mice under terminal anesthesia, infused with 10% formalin, and immersed in the same solution before embedding in paraffin blocks. Five-micrometer sections were stained with H&E to evaluate general morphology. To determine the severity of inflammatory cell infiltration, peribronchial cell counts were performed blind based on a 5 point scoring system described by Duan et al. (2004). Briefly, the scoring system was: 0, no cells; 1, a few cells; 2, a ring of cells 1 cell layer deep; 3, a ring of cells 2–4 cells deep; 4, a ring of cells 4–6 cells deep; 5, a ring of cells >6 cells deep. Mucus-secreting goblet cells were visualized using periodic acid-Schiff (PAS). Goblet cell and airway epithelium were counted by light microscope. To determine the extent of mucus cell production, goblet cell hyperplasia in the airway epithelium was semi-quantified blind using a 5 point grading system described by Kuperman et al. (2002). Briefly, the adopted grading system was: 0, no goblet cells; 1, <15%; 2, 15–30%; 3, 30–45%; 4, >45–60%; 5, >60%. Masson trichrome stain was used for assessment of subepithelial fibrosis using an Image-Pro Plus analysis system (Media Cybernetics, Silver Spring, MD, U.S.). Four to eight specimens from the right lobe were selected. Digital photographs were taken at $440\times$ magnification. The area of collagen deposition (AC) and the perimeter of basement membrane of bronchioles (Pbm) beneath the basement membrane at $20\mu\text{m}$ depth was measured as described by McMillan and Lloyd (2004). Results are expressed as the area of AC per the perimeter of basement membrane of bronchioles ($\text{WAc/Pbm } \mu\text{m}^2/\mu\text{m}$). At least 10 bronchioles were counted from each slide. The adopted grading system was: 0, <5 $\text{WAc/Pbm } (\mu\text{m}^2/\mu\text{m})$; 1, 5–10 $\text{WAc/Pbm } (\mu\text{m}^2/\mu\text{m})$; 2, 10–15 $\text{WAc/Pbm } (\mu\text{m}^2/\mu\text{m})$; 3, 15–20 $\text{WAc/Pbm } (\mu\text{m}^2/\mu\text{m})$; 4, 20–25 $\text{WAc/Pbm } (\mu\text{m}^2/\mu\text{m})$; 5, >25 $\text{WAc/Pbm } (\mu\text{m}^2/\mu\text{m})$. Mean scores of inflammatory cells, goblet cells and collagen deposition were obtained from eight animals. Analyses were performed in a blind fashion, and slides were presented in random order for each examination.

2.8. Data analysis

Numerical data are presented as means $\pm$ S.E.M. Statistical calculations were performed using SigmaStat software (SigmaStat 2.0, SPSS Inc., Chicago, IL, USA), ANOVA and Student–Newman–Keuls multiple comparisons test were used for evaluating differences in respiratory function, number of inflammatory cells, cytokines mRNA expression in lungs and cytokine levels in bronchoalveolar lavage fluid in each group. The non-parametric Mann–Whitney U -test was used to compare infiltration of inflammatory cells, goblet cell hyperplasia and collagen deposition in the airway. Significance was assessed at the $P < 0.05$ level. Experiments were performed independently at least twice; results were qualitatively identical.

3. Results

3.1. Allergen-induced airway hyperresponsiveness to methacholine

The airway responsiveness to intravenous methacholine at the doses of 0, 0.0625, 0.125, 0.25 and 0.5 mg/kg was assessed 24 h after

the last challenge. The responsiveness to methacholine in normal saline-challenged mice showed a weak change in airway resistance (R_L) and lung dynamic compliance (C_{dyn}). However, ovalbumin-challenged mice (OVA-vehicle group) exhibited obvious airway responsiveness with a significant and dose-dependent increase in R_L and decrease in C_{dyn} as compared to saline-challenged mice (Fig. 1 and Table 2). Aerosol pretreatment with BCQB at the doses of 60, 120 and 240 $\mu\text{g}/\text{ml}$ significantly reduced airway responsiveness provoked by methacholine in a dose-dependent manner. Additionally, tiotropium at the dose of 60 $\mu\text{g}/\text{ml}$ also had a more potent effect on reduction of airway responsiveness (Fig. 1 and Table 2).

3.2. Airway eosinophilia and lung inflammation

The total number of inflammatory cells in bronchoalveolar lavage fluid of ovalbumin-vehicle mice was 15-fold greater than that in saline-vehicle mice. Aerosol pretreatment with BCQB at 60, 120 and 240 $\mu\text{g}/\text{ml}$ significantly inhibited the infiltration of inflammatory cells in a dose-dependent manner. Tiotropium at 60 $\mu\text{g}/\text{ml}$ also had a remarkable effect on inhibition of the accumulation of inflammatory cells in airways. Classification of these inflammatory cells indicated that in ovalbumin treated mice, eosinophils, lymphocytes and macrophages in the bronchoalveolar lavage fluid increased 150-, 2- and

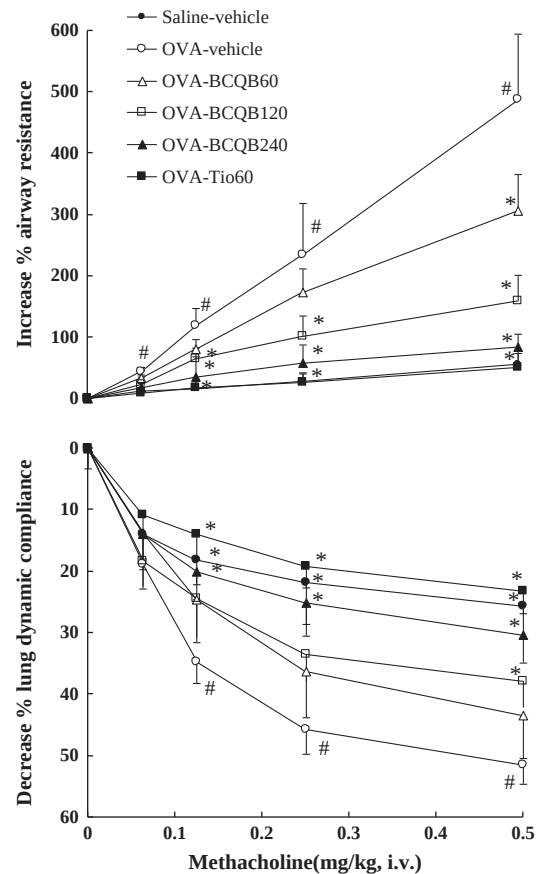


Fig. 1. Dose-dependent response of R_L and C_{dyn} to methacholine. Ovalbumin-sensitized mice were inhaled with bencycloquidinium 60 (BCQB60), 120 (BCQB120) and 240 $\mu\text{g}/\text{ml}$ (BCQB240), tiotropium 60 $\mu\text{g}/\text{ml}$ (Tio60) or vehicle once a day for 7 days. 15 min after aerosol administration of BCQB, Tio or vehicle, mice were challenged with aerosolized antigen or normal saline (NS) for 30 min daily. Twenty-four hours after the last ovalbumin challenge, R_L and C_{dyn} against intravenous application of methacholine was determined as described in Materials and methods. Data are expressed as mean $\pm$ S.E.M. $\#P < 0.05$, versus saline-challenged and vehicle-treated mice ($n = 9$, saline-vehicle group); $*P < 0.05$, Ovalbumin-challenged and BCQB60 ($n = 9$, OVA-BCQB60 group), BCQB120 ($n = 7$, OVA-BCQB120 group), BCQB240 ($n = 8$, OVA-BCQB240 group), Tio ($n = 11$, OVA-Tio60 group) treated mice versus ovalbumin-challenged and vehicle-treated mice ($n = 9$, OVA-vehicle group).

Table 2

Effects of aerosol administration of bencycloquidium and tiotropium on methacholine (MCh)-induced increase of airway resistance (PD_{100}) and reduction of lung dynamic compliance (PD_{25}) in actively sensitized mice. Sensitized mice react at smaller doses of intravenous methacholine, indicating airway hyperresponsiveness. Data are given as mean (95% confidence limits). ^a $P < 0.05$, versus NS-vehicle group; ^b $P < 0.05$, versus OVA-vehicle group.

Groups	n	$R_L PD_{100}$ (95% CI) mg/kg, i.v.	$C_{dyn} PD_{25}$ (95% CI) mg/kg, i.v.
NS-vehicle	9	0.925(0.733–1.166)	0.321(0.226–0.456)
OVA-vehicle	9	0.094(0.080–0.111) ^a	0.109(0.086–0.136) ^a
OVA-BCQB60	9	0.157(0.133–0.186) ^b	0.127(0.107–0.150)
OVA-BCQB120	7	0.227(0.199–0.258) ^b	0.121(0.092–0.160)
OVA-BCQB240	8	0.612(0.499–0.751) ^b	0.238(0.186–0.304) ^b
OVA-Tio60	11	1.610(1.179–2.199) ^b	0.644(0.410–1.012) ^b

3-fold, respectively, as compared to those in saline-vehicle mice (Fig. 2). Pretreatment of ovalbumin-sensitized and -challenged mice with varying doses of BCQB for 7 days significantly decreased eosinophil numbers in a dose-dependent manner, but not lymphocytes. BCQB treatment at 240 μ g/ml, but not tiotropium at 60 μ g/ml significantly inhibited accumulation of macrophages in airways.

3.3. Chemokine, cytokine and matrix metalloproteinase mRNA expression in the lung tissues

To measure cytokine and chemokine mRNA expression in vivo, lung tissues were harvested 24 h after the last ovalbumin challenge. Eotaxin, IL-5, IL-4 and IFN- γ , MMP-9 and TIMP-1 mRNA expression were measured by RT-PCR. As shown in Fig. 3, BCQB significantly inhibited up-regulation of eotaxin, IL-4, IL-5 and MMP-9 mRNA expression in a dose-dependent manner, and treatment of tiotropium 60 μ g/ml also exhibited a marked inhibition on up-regulation of eotaxin, IL-4, IL-5 and MMP-9 mRNA expression (Fig. 3A). BCQB at all dose levels significantly increased IFN- γ mRNA expression in a dose-dependent manner, but only BCQB at 240 μ g/ml augmented TIMP-1 mRNA expression. Tiotropium at 60 μ g/ml also increased IFN- γ mRNA expression, but not TIMP-1 mRNA expression (Fig. 3B).

3.4. Cytokine levels in bronchoalveolar lavage fluid

IL-4 and IFN- γ levels were measured by ELISA. As shown in Fig. 4, in ovalbumin-vehicle mice, IL-4 levels in bronchoalveolar lavage fluid

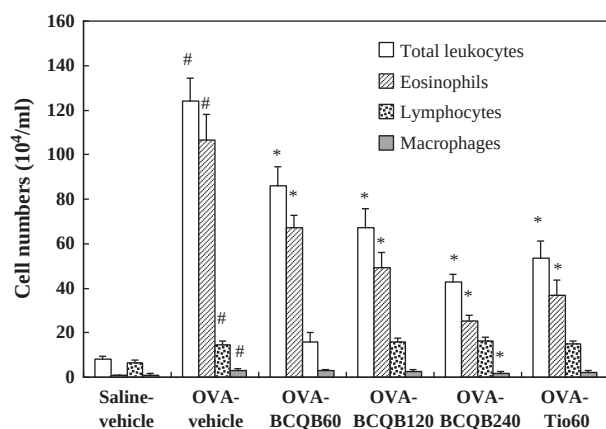


Fig. 2. Effects of bencycloquidium and tiotropium on inflammatory cell levels in bronchoalveolar lavage fluid. Mice were processed as described in Fig. 1, and bronchoalveolar lavage fluid was harvested 24 h after the last ovalbumin challenge. Total inflammatory cell numbers in bronchoalveolar lavage fluid were counted, and cell classification was performed on a minimum of 200 cells to classify eosinophils, neutrophils, and lymphocytes. Data are expressed as mean $\pm$ S.E.M. ^a $P < 0.05$, versus saline-vehicle mice ($n = 9$); ^b $P < 0.05$, OVA-BCQB60 ($n = 8$), BCQB120 ($n = 8$), OVA-BCQB240 ($n = 8$), OVA-Tio60 ($n = 8$) treated mice versus OVA-vehicle ($n = 8$).

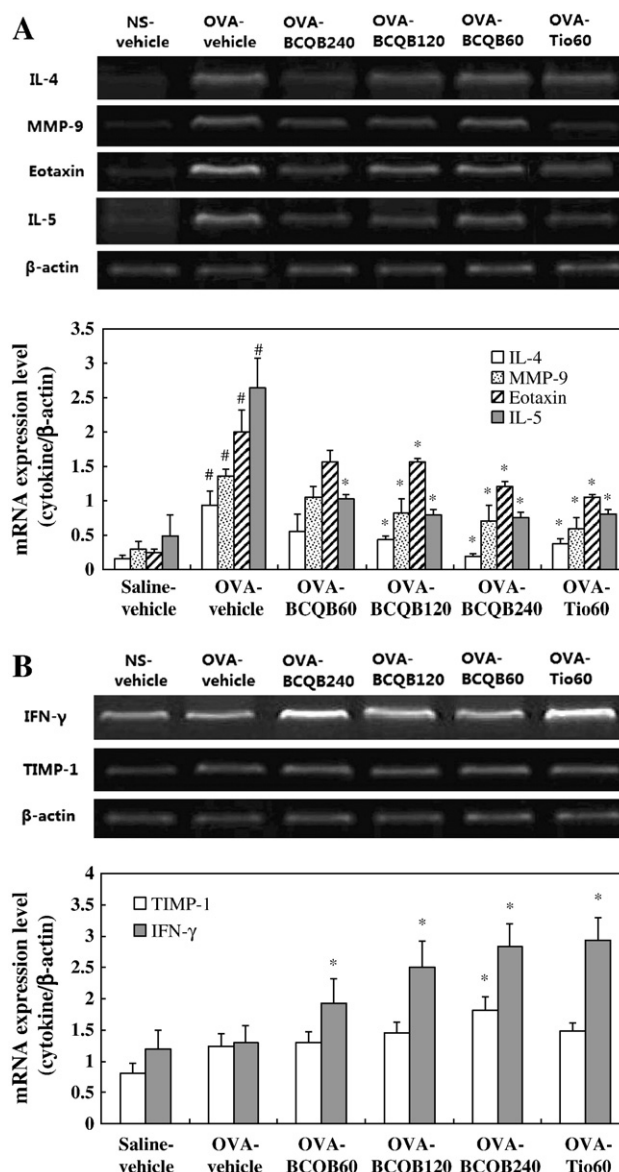


Fig. 3. Effects of bencycloquidium and tiotropium on mRNA expression of eotaxin, IL-4, IL-5, IFN- γ , MMP-9 and TIMP-1 in the lungs of asthmatic mice. Mice were processed and treated as described in Fig. 1. Twenty-four hours after the last ovalbumin challenge, 100 mg of lung tissues from each mouse was harvested for isolation of total RNA, and 1 μ g aliquots of the total RNA sample were used for reverse transcriptional synthesis of first-strand that was used as template for PCR amplification. The PCR product was analyzed by electrophoresis on 1.5% agarose gels and specific bands were digitized using imaging system. PCR reactions were done in triplicates, and the relative expression of mRNAs from independent triplicate experiments was normalized by dividing the eotaxin and cytokine values by the respective β -actin values, and the mean levels of mRNA expression from NS-vehicle mice were defined as 1. A. IL-4, MMP-9, eotaxin and IL-5 mRNA expression; B. IFN- γ and TIMP-1 mRNA expression. Results are shown as mean $\pm$ S.E.M. ^a $P < 0.05$, versus saline-vehicle group; ^b $P < 0.05$, versus OVA-vehicle group.

increased 10-fold compared to those in saline-vehicle mice. However, IFN- γ level in bronchoalveolar lavage fluid did not change significantly compared to saline-vehicle mice. Treatment of ovalbumin-sensitized and -challenged mice with either BCQB or tiotropium significantly increased IFN- γ level and decreased IL-4 level.

3.5. Eosinophil infiltration, mucus production and collagen deposition in lung tissues

Lung tissue was harvested 24 h after the last ovalbumin challenge. Ovalbumin-vehicle mice (Fig. 5B) exhibited an obvious infiltration of

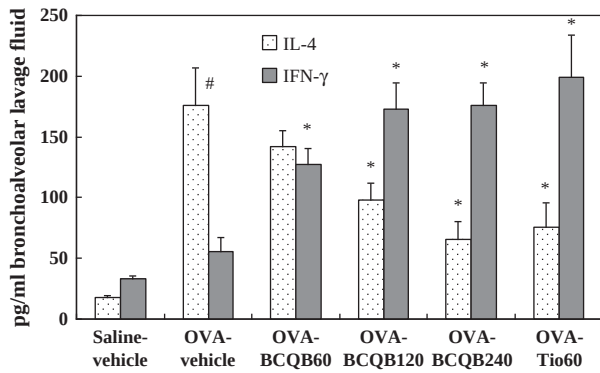


Fig. 4. Effects of bencycloquidum and tiotropium on cytokines levels in bronchoalveolar lavage fluid. Mice were processed and treated as described in Fig. 1, and bronchoalveolar lavage fluid was harvested 24 h after the last ovalbumin challenge. Levels of IL-4 and IFN-γ in bronchoalveolar lavage fluid were analyzed by ELISA. $n=8$ (each group). Results are shown as mean $\pm$ S.E.M. [#] $P<0.05$, versus saline-vehicle group; $P<0.05$, versus OVA-vehicle group.

inflammatory cells into the peribronchiolar and perivascular connective tissues. Goblet cell hyperplasia and mucus hypersecretion within the bronchi of the lungs were also observed. A dramatic increase in both the extent of collagen deposition and intensity of staining as compared with saline-vehicle mice (Fig. 5A). BCQB 120 or 240 $\mu\text{g/ml}$ (Fig. 5D and E) and tiotropium 60 $\mu\text{g/ml}$ (Fig. 5F) markedly attenuated ovalbumin-induced eosinophil infiltration, mucus production and collagen deposition in lung tissues as compared with that in ovalbumin-vehicle mice (Fig. 5B). However, a lower dose of BCQB, 60 $\mu\text{g/ml}$, did not show any obvious effect on eosinophil infiltration, mucus production and collagen deposition in lung tissues (Fig. 5C).

4. Discussion

In the present study, we investigated the effects of BCQB, a selective M_3 receptor antagonist on changes of airway hyperresponsiveness, inflammation and remodeling in a mouse model of asthma. In this model of allergic asthma, antigen sensitization and repeated challenge induced airway hyperresponsiveness, inflammation, goblet cell

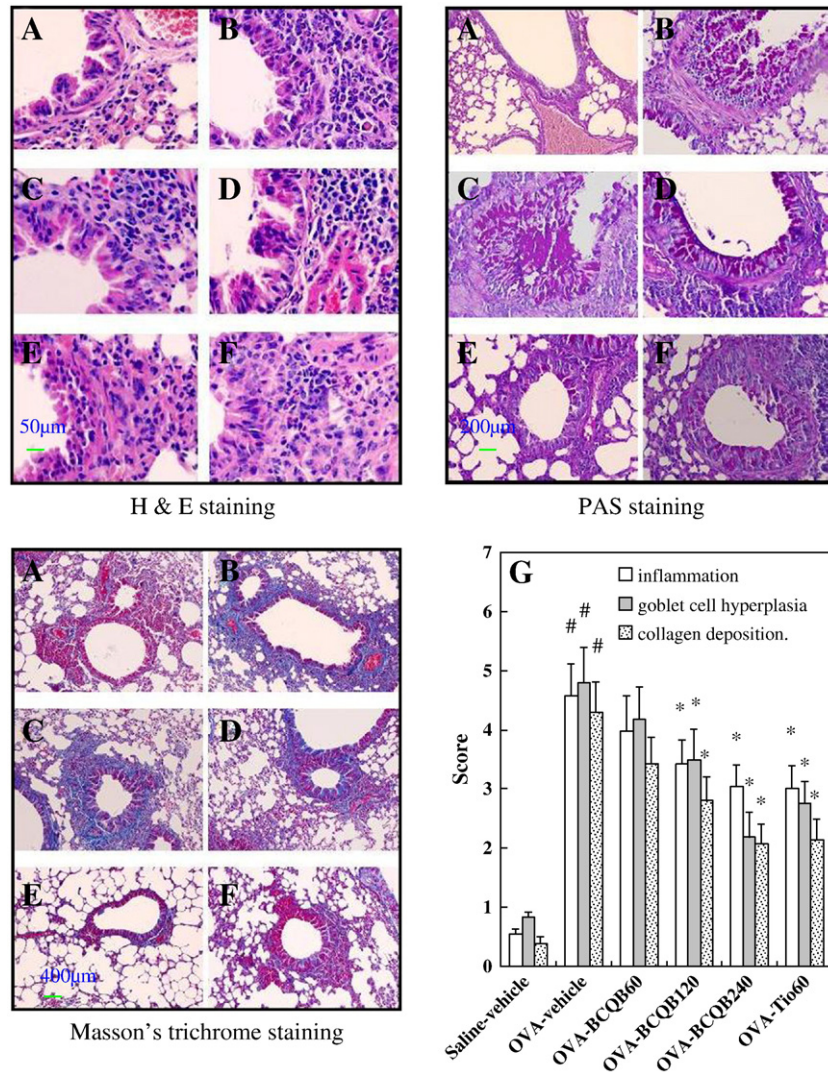


Fig. 5. Effects of bencycloquidum and tiotropium on eosinophil infiltration, mucus secretion and collagen deposition (A–F) and were performed 24 h after the last allergen challenge. A: Saline-vehicle group; B: OVA-vehicle group; C: OVA-BCQB60 group; D: OVA-BCQB120 group; E: OVA-BCQB240 group; F: OVA-Tio60 group. Semi-quantitative analysis of inflammatory cell infiltration, mucus production and collagen deposition (G) in lung sections were performed as described in Materials and Methods. Lung tissues were fixed, sectioned at 5 μm , and stained with H&E for tissue eosinophils infiltration, periodic acid-Schiff for mucus production (red are positive for mucus) or Masson's trichrome for collagen deposition (blue are positive for collagen deposition). Results are shown as mean $\pm$ S.E.M, $n=8$ in each group. [#] $P<0.05$, versus saline-vehicle group; $P<0.05$, versus OVA-vehicle group.

hyperplasia, collagen deposition, and an increase in mRNA expression of eotaxin, IL-4, IL-5 and MMP-9, but did not decrease in mRNA expression for IFN- γ and TIMP-1. A parallel change in the protein levels of IL-4, and IFN- γ in the bronchoalveolar lavage fluids was also observed. These changes were reversed after BCQB and tiotropium treatment. Additionally, BCQB significantly increased IFN- γ mRNA expression in lung tissue and IFN- γ protein level in the bronchoalveolar lavage fluids. BCQB at higher dose also elevated TIMP-1 mRNA expression in lung tissue. Tiotropium could significantly increase IFN- γ mRNA expression in lung tissue and IFN- γ protein level in the bronchoalveolar lavage fluids, but not the mRNA expression of TIMP-1 in lung tissue. Collectively, it appears that endogenous acetylcholine, acting on muscarinic receptors, plays a broad role in the pathology of allergic airways disease. Therefore, targeting these effects with anticholinergics may provide a novel therapeutic approach to the treatment of asthma-associated pathology.

Accumulating evidence has demonstrated that acetylcholine and its synthesizing enzyme, choline acetyltransferase, are present not only in airway nerves, but are localized to epithelial and endothelial cells, smooth muscle cells, lymphocytes, macrophages, mast cells, eosinophils and neutrophils (Gwilt et al., 2007; Proskocil et al., 2004; Wang et al., 2001; Kawashima and Fujii, 2000). Acetylcholine is known to have different immunological effect on cells. It is predominantly pro-inflammatory for lymphocytes and epithelial cells, while anti-inflammatory for mast cells and macrophages. It can be either pro- or anti-inflammatory for monocytes, and may have variable effect on neutrophils and eosinophils (Gwilt et al., 2007). Furthermore, rigorous investigation has now revealed that most inflammatory cells express functional muscarinic receptors (Gosens et al., 2004; Gwilt et al., 2007; Wessler and Kirkpatrick, 2008). These findings suggest that acetylcholine can regulate inflammatory processes by paracrine and/or autocrine mechanisms. Tiotropium is a muscarinic receptor antagonist and a potent agent to treat patients with bronchial asthma as well as COPD. Recent evidence has demonstrated that inhaled administration of this drug significantly reduced airway inflammation and airway hyperresponsiveness in several animal and disease models, e.g. in a mouse model of asthma (Ohta et al., 2010), allergen-induced airway remodeling in a guinea pig model (Bos et al., 2007), and cigarette smoke-induced pulmonary inflammation in mice (Wollin and Pieper, 2010). However, effect of a selective M₃ receptor antagonists on allergen or cigarette smoke-induced asthma in mice model have not been reported to date. In the present study, we evaluated for the first time, a selective M₃ receptor antagonist, BCQB, for its ability to affect pulmonary inflammation by comparing to tiotropium.

It is known that Th2 cells play an important role in the modulation of allergic airway inflammation (Wegmann, 2009; Bowen et al., 2008), and immune imbalance with a skewing toward a Th2 bias is an important character in asthma. Our present data show that BCQB and tiotropium significantly inhibited mRNA expression of eotaxin, IL-4, and IL-5 in lung tissues in a dose-dependent manner. In contrast, IFN- γ , a Th1 cytokine, was increased by BCQB and tiotropium. In addition, BCQB and tiotropium also significantly decreased the levels of IL-4 and increased the levels of IFN- γ in bronchoalveolar lavage fluids. These results indicate that BCQB and tiotropium reversed the imbalance between Th1/Th2 cytokines in asthmatic mice.

Eosinophils are key effector cells for the pathogenesis of allergic inflammation. Our present findings showed that BCQB and tiotropium inhibited eosinophil infiltration into the airways, as shown by a significant decrease in total cell counts and eosinophil counts in bronchoalveolar lavage fluid. Similarly, airway tissue infiltration of eosinophil was also attenuated, as revealed by a significant reduction in inflammatory cell infiltration in histological examination. Eosinophil transmigration into the airways is a multistep process that is orchestrated by Th2 cytokines such as IL-5 and coordinated by specific chemokines such as eotaxin in combination with adhesion molecules such as VCAM-1 and VLA-4 (Takatsu and Nakajima, 2008; Bisset and Schmid-Grendelmeier, 2005).

Our results showed that mRNA expression of eotaxin and IL-5 in lung tissue from BCQB or tiotropium-treated mice was substantially reduced. Taken together, the observed reduction in airway eosinophilia by BCQB or tiotropium may result from a combined effect of the reduction of Th2 cytokine production and eotaxin formation.

Airway remodeling is a major cause for irreversible asthmatic airflow restriction. MMPs are the key enzymes responsible for extracellular matrix degradation (Fernandes et al., 2006). And MMP-9 is the most relevant to asthma among the 23 human MMPs (Kelly et al., 2000; Lee et al., 2001). Although the mechanism is still under investigation, the imbalance between MMP-9 and TIMP-1 is considered a primary explanation of airway remodeling. In terms of the potential effects of MMP-9 on airway inflammation in asthma, in vitro studies have demonstrated that MMP-9 may be important in mediating migration of eosinophils across the basement membrane of blood vessels (Okada et al., 1997). To explore the potential role of MMP-9 in vivo in mediating eosinophilic airway inflammation and airway responsiveness, previous studies have been carried out using MMP-9-deficient mice in acute allergen-induced asthma. These studies have produced different results regarding the role of MMP-9 in mediating eosinophilic airway inflammation and airway responsiveness (Cataldo et al., 2002; Corry et al., 2004; McMillan et al., 2004; Vermaelen et al., 2003). Our results demonstrated a dramatic reduction in mucus production with less goblet cell hyperplasia and collagen deposition in lung tissues in BCQB or tiotropium-treated mice as compared with untreated control. In addition, the mRNA expression of MMP-9 in the lung tissues from BCQB or tiotropium-treated mice was significantly reduced, and the mRNA expression of TIMP-1 was substantially increased in BCQB-treated mice.

Airway hyperresponsiveness to direct (histamine and methacholine) and indirect (exercise, cold air, hyperventilation, and AMP) challenges is a universal and defining feature of asthma. The inflammatory mediator release and airway remodeling during the allergic inflammation play a critical role in airway hyperresponsiveness development (Cockcroft and Davis, 2006; Bergeron et al., 2009). Our data show that BCQB significantly inhibited ovalbumin induced airway hyperresponsiveness to methacholine in a dose-dependent manner. It has been established that IL-5 and eotaxin play a critical role in airway hyperresponsiveness by mobilizing and activating eosinophils. Proinflammatory products such as major basic protein and cysteinyl-leukotrienes will be released, which are closely associated with airway hyperresponsiveness (Mattes and Foster, 2003). In addition, IL-5, eotaxin and IL-13 have been shown to induce airway hyperresponsiveness in mouse asthma models in which cysteinyl-leukotrienes have been implicated to play a major role in airway hyperresponsiveness (Eum et al., 2003). Moreover, airway remodeling refers to airway structural changes which also play a critical role in persistent airway hyperresponsiveness. Changes include epithelial alteration, subepithelial fibrosis, increased smooth muscle mass, goblet cell and mucous gland hyperplasia (Bergeron and Boulet 2006; Cockcroft and Davis, 2006). As such, the observed reduction of airway hyperresponsiveness by BCQB may be associated with reduction in expression of IL-5, eotaxin and MMP-9, tissue eosinophilia, goblet cell hyperplasia and subepithelial fibrosis.

A recent comprehensive review and meta-analysis of 17 randomized controlled trials of inhaled muscarinic antagonists (mainly ipratropium bromide or tiotropium bromide) in COPD patients has revealed that these drugs are associated with a significantly increased risk of cardiovascular death, myocardial infarction, or stroke among patients exposed to more than 6 months of therapy (Singh et al., 2008). In contrast, a most recent clinical study has denied this uncertain systematic review and meta-analysis. The resulting database includes 30 trials in which 10,846 patients were randomised to tiotropium and 8699 to placebo. An analysis of these trials indicated that tiotropium was associated with a reduction in the risk of all cause mortality, mortality from cardiovascular disease, and cardiovascular

events (Celli et al., 2010). Nonetheless, the proposed benefit of using a selective M₃ antagonist may reduce the side effect profile while maintaining therapeutic efficacies. Inhalable M₃-selective compounds may spare M₂-cardiac receptors and reduce the risks of cardiovascular events associated with the long-term treatment of these agents. A recent study showed that muscarinic receptor expression did not differ between animal strains, whereas M₂R expression was 70-fold higher than M₃R in the heart (Tuomi et al., 2010). Additionally, blockade of M₂R induced a significant tachycardia, while blockade of M₃R only have a weak effect (Wang et al., 2004). The selective M₃R antagonist darifenacin eliminated atrial tachycardia and fibrillation induced by electrical stimulation (Tuomi et al., 2010). BCQB, as a selective M₃R antagonist, compared with ipratropium bromide or tiotropium bromide, at high concentration (10⁻⁵ M) resulted in a slight increase in myocardial constriction, induced less tachycardia in the isolated guinea pig atrium. In guinea pigs in vivo, BCQB induced a smaller and more transient increase in heart rate than tiotropium at comparable supratherapeutic doses (unpublished data). These results indicate that BCQB may be a favorable cardiovascular safety profile, though tiotropium is more effective in controlling pulmonary inflammation in the present study.

In the present study, a new M₃ receptor inhibitor, BCQB, was studied for the first time for its effect on airway inflammation and hyperresponsiveness. Our results showed that BCQB effectively reduced ovalbumin-induced Th2 cytokine production, pulmonary eosinophilia, MMP-9 expression in lung tissues, mucus hypersecretion, and airway hyperresponsiveness in a mouse asthma model. These findings may provide a novel therapeutic use of M₃ receptor inhibitor in the treatment of asthma.

Competing interests

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.ejphar.2011.01.024.

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